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Virology 340 (2005) 277 – 284

VIROLOGY

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Characterization of antibody responses to purified HIV-1 gp120 glycoproteins fused with the molecular adjuvant C3d

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Received 23 February 2005; returned to author for revision 14 June 2005; accepted 20 June 2005
Available online 26 July 2005

Abstract

The HIV-1 exterior envelope glycoprotein gp120 binds receptor (CD4) and co-receptors (CCR5/CXCR4) and is a major target for neutralizing antibodies. The two functionally conserved regions of gp120 involved in receptor binding are conformational in nature. It is likely that the elicitation of neutralizing antibodies to these targets will benefit by presentation of these sites to the humoral immune system under physiologic conditions. Initially, we investigated the ability of the molecular adjuvant C3d to enhance antibody responses to variant gp120 glycoproteins in phosphate-buffered saline (PBS). We utilized a gp120 variant glycoprotein deleted of N- and C-terminal sequences (gp120ΔC1/C5) originally designed to eliminate immunodominant, non-neutralizing epitopes and characterized this protein when fused to two C3d elements (gp120ΔC1/C5$(C3d)_2$). In PBS, the gp120ΔC1/C5$(C3d)_2$ proteins are able to elicit gp120 binding antibodies more efficiently than gp120 lacking C3d moieties. We then asked if we could observe C3d-enhanced immunogenicity of gp120 in the presence of the classical oil-in-water adjuvant, Ribi. In the presence of the Ribi, which contains the TLR-4 agonist monophospholipid A (MPL), antibodies elicited by the gp120ΔC1/C5$(C3d)_2$ were of higher titer than those elicited by the identical protein in PBS. To determine if the elicited secondary response was due to a synergy between the C3d repeats and the Ribi, we then inoculated gp120ΔC1/C5 protein in Ribi and observed that similar titers of anti-gp120 antibodies were elicited in comparison to the gp120ΔC1/C5$(C3d)_2$ protein also inoculated in Ribi adjuvant. In Ribi, there was a small but consistent increase in gp120-specific antibody titer of a gp120ΔC1/C5$(C3d)_2$ prime followed by two gp120ΔC1/C5 boosts compared to three inoculations of either the gp120ΔC1/C5 proteins or the gp120ΔC1/C5$(C3d)_2$ proteins alone. We conclude that the molecular adjuvant C3d demonstrates utility in conditions where physiologic presentation of native protein structures is desired, but may have less benefit in the context of a relatively potent protein adjuvant such as Ribi.
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Keywords: HIV-1; Glycoprotein; Molecular adjuvant

Introduction

The HIV-1 exterior envelope glycoprotein gp120 mediates receptor binding by first binding to cellular CD4 and then, following conformational changes, binding to the chemokine receptor co-receptors, either CCR5 or CXCR4 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). Due to its necessary location on the surface of the virus to engage receptor, gp120 is also a major target for neutralizing antibodies (Wyatt and Sodroski, 1998; Wyatt et al., 1998). Since most effective anti-viral vaccines that protect against viral challenge do so in part, or wholly, by neutralizing antibody responses (Pantaleo and Koup, 2004), the elic-

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0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2005.06.034

itation of such responses against HIV remains a major goal for vaccine development. Means to enhance ("adjuvanate") antibody responses to gp120 are therefore of great interest. The use of adjuvant to better elicit antibody responses to soluble proteins is a proven methodology; however, only one adjuvant (alum) is currently approved for human use. Interest in using the so-called molecular adjuvants, with more specific and molecularly defined modes of action, has grown over recent years. One relatively well-studied molecular adjuvant is a component of the complement pathway known as C3d, which, as a fusion protein, has been shown to possess adjuvant properties (Dempsey et al., 1996). In a series of papers, Ross and colleagues have shown that proteins, including the HIV-1 envelope glycoproteins, fused with C3d repeats and expressed in vivo from plasmid DNA demonstrate enhanced immunogenicity and, in one case, improved neutralizing antibody responses (Bower et al., 2004a, 2004b; Green et al., 2003; Liu et al., 2004; Ross et al., 2001). Raising anti-Env antibody responses under physiologic, non-denaturing conditions might increase the likelihood of eliciting responses to the conserved, conformation-dependent neutralization epitopes present on the HIV-1 envelope glycoproteins. In addition, it is possible that C3d repeats will synergize with classical oil-in-water adjuvants such as Ribi. Besides properties of protein deposition, the Ribi formulation used in mice activates the immune system by direct interaction of its monophospholipid A (MPL) component with toll-like receptor 4 (TLR-4) found on antigen-presenting cells such as dendritic cells (Baldridge et al., 2004; Miller et al., 2005).

The complement protein, C3d, plays a key role in linking the innate immune system to antigen-specific antibody formation. In the absence of antibody, foreign proteins or microorganisms can activate the complement system though the alternative pathway by proteolysis of the complement protein, C3, to generate C3b. Activated C3b contains a highly reactive internal thiol ester, which can covalently attach to available protein amine groups or to hydroxyl groups present on carbohydrate-containing glycoproteins of the invading microorganism. Once covalently linked to the foreign protein, C3b undergoes a further proteolytic modification to generate C3d, which results in a C3d-tagged protein. The C3d-tagged proteins have the capacity to bind complement receptors (CD21, previously designated CR2) expressed on the surface of B cells (Molina et al., 1992). In this model, B-cell clones possessing surface IgM (sIgM) that recognize an epitope on the C3d-tagged protein become preferentially activated by the cross-linkage created by the C3d-tagged protein being bound simultaneously to CD21 (via the C3d moiety) and to sIgM (via epitopes on the foreign protein; for example, gp120-C3d; see Fig. 1). Signaling to downstream intracellular pathways is mediated by the CD21-associated molecule, CD19.

The initial observations of Dempsey et al. exploited the ability of C3d to target a model protein (hen egg white lysozyme; HEL) to complement receptors on B cells (and perhaps other immune cell types) to enhance the immunogenicity of HEL-C3d fusion proteins possessing one, two or three repeats of the C3d moiety. Mice immunized with HEL-C3d fusion proteins in phosphate-buffered saline as a primary inoculation, followed by a HEL protein boost in adjuvant, elicited IgG antibodies at a level comparable to that achieved with HEL as a prime in complete Freund's adjuvant (CFA) (Dempsey et al., 1996). Experimentally, the apparent co-ligation of CD21/19 and sIgM lowers occupancy of the number of antigen receptors required for B-cell activation by two orders of magnitude and may be the mechanism by which sIgM-CD21 cross-linking increases B-cell activation. Since the follicular dendritic cells (FDCs) also express CD21 molecules, they too may be activation targets of the C3d-tagged immunogens. However, a more recent study observed enhancement of IgG secondary antibody responses to C3d-ligated proteins in CD21/35-deficient mice and suggested that C3d may also act via an as-yet-to-be-identified CD21-independent pathway (Haas et al., 2004). Therefore, it is possible that CD21-negative cells may also participate in the immune-enhancing effects of C3d during natural infection or following immunization.

In any case, the use of C3d as a "molecular adjuvant" thus provides an attractive means of eliciting a high-titer antibody response under conditions in which it may be desirable to avoid the use of (potentially) denaturing adjuvants, such as preservation of the quaternary structure of a complex protein immunogen. A recent study has suggested that three repeats of C3d appended to trimeric HIV-1 envelope glycoproteins expressed by injection of plasmid DNA more effectively elicited neutralizing antibodies to primary isolates than vectors encoding for only the envelope glycoproteins (Bower et al., 2004b).

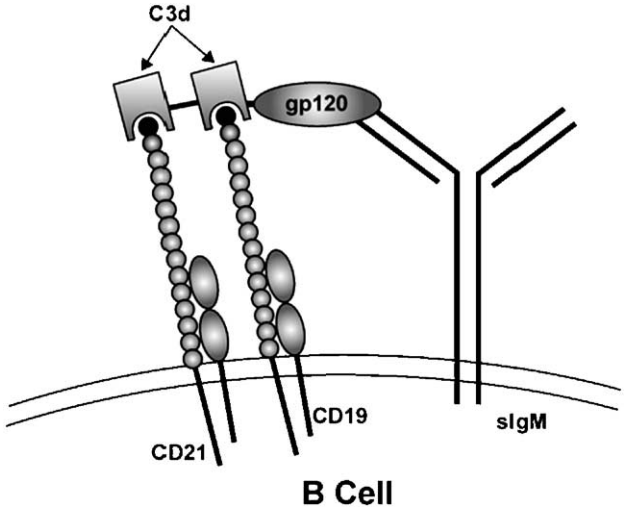

Fig. 1. Schematic depiction of a gp120ΔC1/C5(C3d)$_2$ fusion protein interacting with two CD21 molecules (CR2) on the surface of a gp120-specific B cell. The molecule CD19 is associated with CD21 as part of a signaling complex. Co-ligation of CD21 and surface IgM (sIgM) lowers the apparent amount of antigen required for B-cell activation, although other CD21-independent pathways may exist.

In the study by Haas et al., there was no enhanced antibody responses to gp120 proteins fused to three repeats of C3d and inoculated two times into wild-type C57Bl/6 mice (Haas et al., 2004). In contrast, if the gp120-C3d$_3$ was expressed from DNA, or was inoculated into CD21-deficient "knockout" mice as protein, increased antibody responses were better elicited by the gp120-C3d$_3$ fusion as compared to wild-type gp120. In our current study, we sought to determine if enhanced immunogenicity to gp120 could be observed with two fused repeats of C3d inoculated at a lower dose into wild-type Balb/c mice, and if enhancement would persist over repeated inoculations. According to the model proposed by Dempsey et al., such gp120-C3d fusion proteins would be capable of binding to CD21 on the surface of B cells and FDCs (Fig. 1). We then asked if there were a difference in outcome if we primed with the gp120 fused to C3d and then boosted with proteins either containing or lacking C3d. Finally, we determined if we could still detect enhanced IgG antibody responses to gp120-C3d fusion proteins in the presence of classical adjuvant.

We initially examined the effects of fusing two repeats of C3d to a gp120 variant glycoprotein harboring deletions at the N- and C-termini (gp120ΔC1/C5(C3d)$_2$) on protein expression, folding and immunogenicity. The gp120ΔC1/C5 construct was originally designed to eliminate immunodominant, non-neutralizing determinants present on gp120 (Grundner et al., 2004) and was used as a control protein in the current study. Immunogenicity experiments with the two proteins in PBS demonstrated that, indeed, the two C3d repeats fused to gp120 glycoproteins significantly enhanced the elicitation of secondary IgG antibodies to gp120. We then examined the immunogenicity of gp120ΔC1/C5(C3d)$_2$ proteins in the classical oil-in-water adjuvant, Ribi. Ribi adjuvant contains the TLR-4 agonist MPL as a major immune-activating component and we sought to determine if there is a detectable additive or synergistic effect in eliciting anti-gp120 IgG secondary responses between the two types of adjuvants targeting distinct innate immune pathways. We report that, as opposed to a clear enhanced effect of the fused C3d repeats on gp120 immunogenicity in PBS, in the classical oil-in-water adjuvant Ribi, there was less observable benefit of fused C3d repeats on the elicitation of gp120-directed IgG antibodies. In Ribi adjuvant, when the gp120ΔC1/C5(C3d)$_2$ proteins were used as a prime, followed by two gp120ΔC1/C5 boosts, there was a slight but consistent increase in elicited anti-gp120 titers as determined by anti-gp120 IgG ELISA.

Results

Protein characterization and conformational analysis of gp120 and C3d$_2$ moieties

The purified gp120ΔC1/C5(C3d)$_2$ fusion proteins were analyzed to verify that native conformation was maintained in both the gp120 and C3d portions of the molecule by immunoprecipitations. The gp120ΔC1/C5 protein was analyzed in parallel as either a positive or negative control. Both the gp120ΔC1/C5 protein and the gp120ΔC1/C5(C3d)$_2$ fusion proteins were recognized by each of the conformational antibodies F105 and 17b, indicating that the gp120 domains were in a native conformation following purification (Fig. 2B). CD4 recognition was assessed indirectly by induction of 17b binding. AIDS patient sera were used in antibody excess to determine the total amount of gp120 that could be efficiently immunoprecipitated in the assay.

Besides noting the increased apparent molecular weight of the (C3d)$_2$ fusion proteins in SDS gels (Fig. 2A), we performed two assays to determine both that the C3d elements were present in the fusion protein and that the C3d moieties were functional in terms of their ability to bind to CD21. To simply detect the presence of the C3d moieties, the fusion proteins were specifically immunoprecipitated by both anti-mouse-C3 and anti-human-C3d polyclonal antisera (Fig. 2B). To confirm that C3d could recognize CD21 in the context of the fusion protein, a binding assay was performed with Raji B cells, which express human CD21 on their cell-surface. Previously, these cells had been shown to bind fusion proteins containing murine C3d identical to the

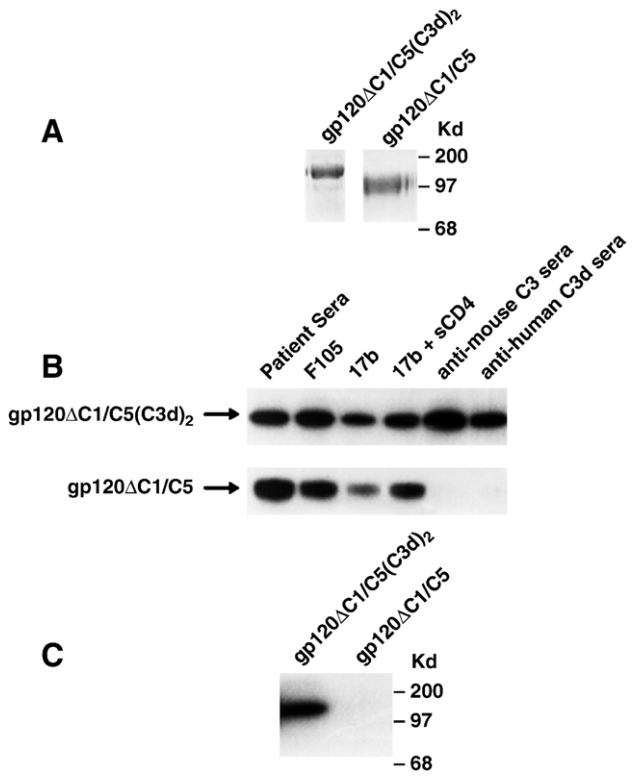

Fig. 2. Analysis of the gp120ΔC1/C5 and gp120ΔC1/C5(C3d)$_2$ glycoproteins expressed from *Drosophila* S2 cells. Panel A, a Coomassie blue-stained SDS gel of recombinant proteins purified by F105 affinity chromatography; panel B, immunoprecipitations with the designated monoclonal/CD4 and polyclonal antibodies; panel C, iodinated gp120ΔC1/C5 and gp120ΔC1C5(C3d)$_2$ binding to CD21$^+$ Raji B cells followed by immunoprecipitation and autoradiography.

sequences used in the current study (Dempsey et al., 1996). In this assay, the ^{125}I-labeled gp120ΔC1/C5 did not bind to the Raji cells at a detectable level, whereas the ^{125}I gp120ΔC1/C5(C3d)$_2$ glycoprotein efficiently bound to the CD21$^+$ cells (Fig. 2C), demonstrating that the fusion protein was targeted to these cells by properly folded C3d moieties. Taken together, these data strongly suggest that each element of the gp120ΔC1/C5(C3d)$_2$ fusion protein is folded in a functional, native conformation.

Analysis of sera from mice inoculated with gp120 possessing or lacking C3d

To determine the effects of C3d in a physiologic buffer and in the absence of a classical adjuvant, we analyzed the levels of anti-gp120 IgG antibodies elicited by the purified gp120 glycoproteins, either lacking or possessing the two C3d repeats, following a second, third and fourth inoculation in PBS. Sera derived after each inoculation of the gp120ΔC1/C5(C3d)$_2$–PBS proteins exhibited higher titers of anti-gp120 antibodies when compared to sera elicited by the gp120ΔC1/C5 proteins lacking the C3d repeats, as determined by IgG ELISA (Fig. 3).

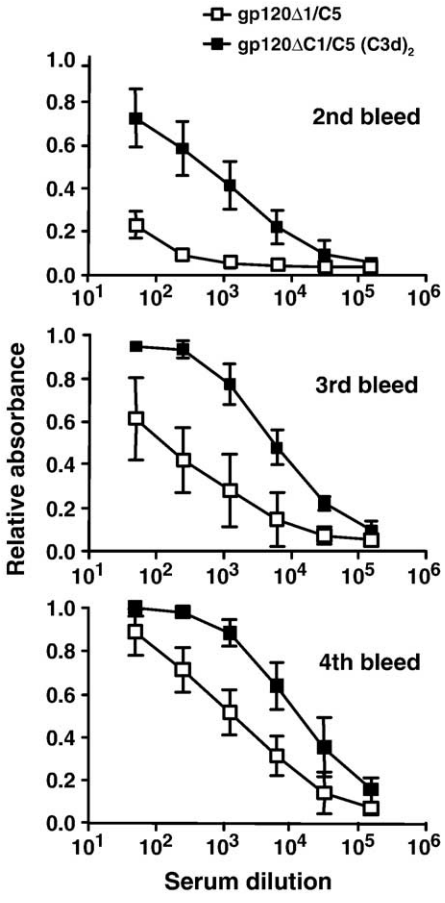

Fig. 3. ELISA analysis of pooled sera from mice inoculated with gp120ΔC1C5 in PBS as compared to mice inoculated with gp120ΔC1/C5(C3d)$_2$ in PBS after 2, 3 and 4 inoculations. Test bleeds were obtained 7–10 days following each inoculation. Error bars indicate range of values obtained for duplicate samples.

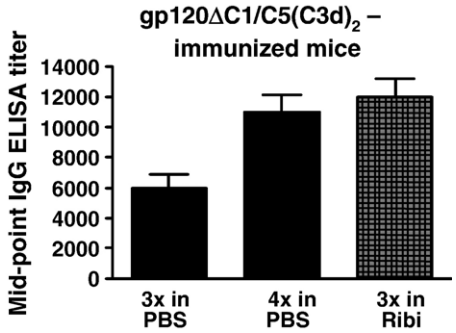

Fig. 4. Half-maximal binding to gp120 of IgG from pooled sera of mice inoculated with gp120ΔC1/C5(C3d)$_2$ in PBS compared to inoculation in Ribi adjuvant. Error bars indicate range of values obtained for duplicate samples.

We then sought to determine if the gp120ΔC1/C5(C3d)$_2$ proteins were more immunogenic in adjuvant such as the commercially available, MPL-containing adjuvant Ribi. Since MPL is a TLR-4 agonist, we reasoned that enhanced immunogenicity might be observed if both CD21 and TLR-4 pathways were targeted by the gp120ΔC1/C5(C3d)$_2$ proteins. As seen in Fig. 4, the gp120ΔC1/C5(C3d)$_2$ in Ribi did elicit higher-titer anti-gp120 IgG antibodies compared to animals inoculated with the identical protein at the identical dose and route, but administered in PBS. Shown are the half-maximal binding values determined by ELISA. After 4 inoculations, the half-maximal titer of the gp120ΔC1/C5(C3d)$_2$ in PBS did approach the levels of anti-gp120 IgG antibodies elicited by 3 inoculations of the proteins in Ribi adjuvant (Fig. 4). Four inoculations of the proteins in Ribi did not further boost the anti-gp120 IgG titers (not shown).

These data did not clearly define if there was an additive affect between the C3d fusion protein and the Ribi, or if the Ribi components themselves were the major factor contributing to the enhanced elicitation of antibodies by the gp120ΔC1/C5(C3d)$_2$ proteins. We then inoculated 3 groups of mice with selected proteins, all emulsified in Ribi adjuvant. Group A mice were inoculated three times with gp120ΔC1/C5 lacking the C3d repeats, group B mice received one priming dose of gp120ΔC1/C5(C3d)$_2$ and then two boosts of gp120ΔC1/C5 protein and group C were inoculated with three equivalent doses of gp120ΔC1/C5(C3d)$_2$ glycoproteins. Following the third inoculation, the sera were analyzed by ELISA and, as seen in Fig. 5, all three groups displayed a similar pattern of increasing levels of anti-gp120 IgG antibodies following each inoculation. However, the mice primed with the gp120ΔC1/C5(C3d)$_2$ glycoproteins and boosted with the gp120ΔC1/C5 proteins (group B) had roughly a 2- to 3-fold higher titer of gp120-binding IgG antibodies at half-maximal binding compared to the gp120ΔC1/C5 priming and boosting (group A). In this assay, a slightly greater maximal absorbance at the

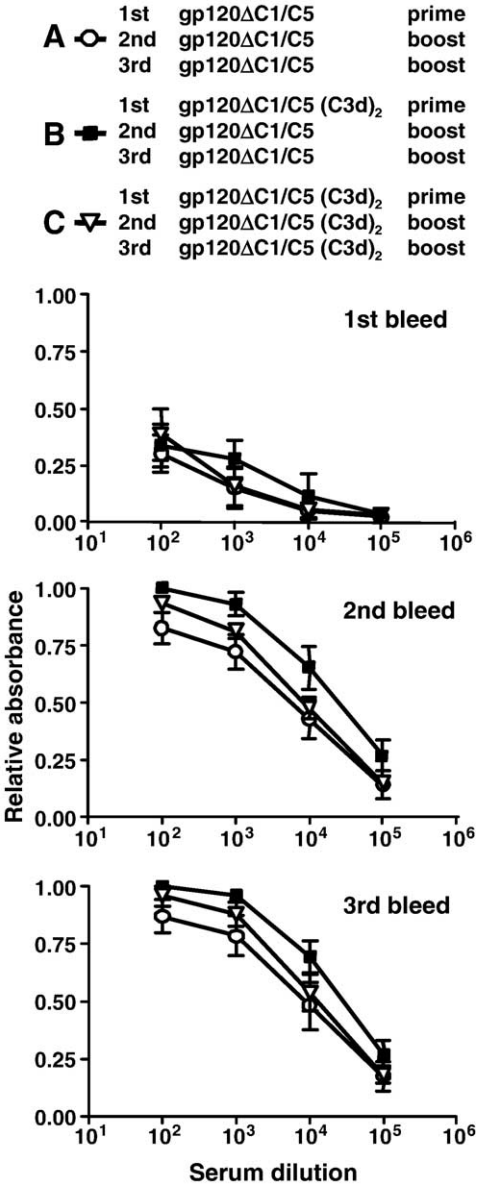

Fig. 5. ELISA analysis of pooled sera from mice inoculated with one prime and two boosts of recombinant proteins. Bleeds 1, 2 and 3 were obtained 7–10 days after each inoculation. Group A, gp120ΔC1C5 prime and boost; group B, gp120ΔC1/C5$(C3d)_2$ prime and gp120ΔC1/C5 boosts; group C, gp120ΔC1/C5$(C3d)_2$ prime and boosts. Error bars indicate range of values obtained for duplicate samples.

highest serum concentrations tested could also be observed for group B compared to group A after either two or three inoculations. We also observed that in this experiment, near maximal IgG titers were elicited by a single protein prime followed by one protein boost in the Ribi adjuvant (Fig. 5).

Discussion

As more complex and hopefully improved HIV-1 Env-based vaccine candidates are developed, it is important to define the optimal means to elicit antibodies against these Env mimetics. Since most forms of unmodified, monomeric gp120 have been disappointing in terms of eliciting broadly neutralizing antibodies in animals and particularly human clinical trials (Flynn et al., 2005), many investigators have sought to develop modified immunogens that may better mimic the conserved regions present on the native viral spike and thereby better elicit broadly reactive neutralizing antibodies. Many investigators, including ourselves, are developing second generation trimeric immunogenic platforms which appear to be the most promising candidates to more efficiently present conserved epitopes to the host immune system (Binley et al., 2000; Gao et al., 2005; Sanders et al., 2002; Srivastava et al., 2003a, 2003b; Yang et al., 2000a, 2000b, 2001, 2002). In this study we have used a candidate "first generation" modified monomeric gp120 Env glycoprotein to study the effects on the elicitation of gp120-directed binding antibodies of two types of adjuvants: the so-called molecular adjuvant C3d and a more classical oil-in-water adjuvant, Ribi. The original rationale for the gp120 modifications was to remove immunodominant non-neutralizing epitopes on the C- and N-terminus of gp120. We have previously determined these regions are not exposed on the trimeric spike (Pancera and Wyatt, 2005; Wyatt et al., 1997) and we sought to determine if these modifications would better elicit antibodies directed against less immunogenic neutralizing determinants still available on the modified monomer and present on the functional Env spike (Grundner et al., 2004; Wyatt et al., 1997). In preliminary data this candidate immunogen did elicit detectable neutralization of the homologous, lab-adapted strain HXBc2 and another relatively easy to neutralize strain, MN, but not the primary isolate 89.6 as determined by a pseudotype viral entry/neutralization assay. Due to the limits of sera, confirmatory assays could not be performed within the scope of this study.

In this report, and as anticipated from most earlier studies, we have clearly shown that the C3d fusion moieties enhance IgG secondary antibody responses to gp120 proteins in physiologic saline. It is important to establish that C3d can enhance gp120 immunogenicity in physiologic buffer, as this was not observed with gp120-$C3d_3$ proteins in the context of wild-type C57/Bl6 mice (Haas et al., 2004). However, since these investigators could observe a benefit when the same construct was expressed in C57/Bl6 mice from DNA, it suggested that perhaps, at a lower dose, such a benefit might be more apparent. In part, this was why we performed our studies at a lower dose than the previous study, an aspect that may deserve further investigation.

We also report that in the presence of the classical adjuvant Ribi, which is designed to both enhance antigen deposition and activate immune danger signals through TLR-4, there was less benefit of the fused C3d repeats on gp120 immunogenicity. The reduced benefit of C3d in Ribi adjuvant may be due to the activation of CD21-independent pathways also targeted by C3d (Haas et al., 2004) that are already maximally activated in the presence of Ribi. We

conclude that the addition of C3d repeats to candidate gp120 or gp140 immunogens does have benefit in the presence of physiologic buffers such as PBS, even when the immune presentation performed in this manner lacks the "depot" properties of an oil-in-water adjuvant emulsion provided by the Ribi formulation. The use of such a molecular adjuvant could be of benefit for enhancing the immune responses elicited by DNA inoculation, as has been demonstrated by Ross and colleagues (Bower et al., 2004a, 2004b; Ross et al., 2001). As also suggested by these investigators, the use of C3d-mediated immune enhancement could also be of benefit to better maintain the native conformation of recombinant HIV Env trimeric proteins. The native structure of these proteins is normally assessed under physiological conditions and it may be important, if not critical, to keep these trimeric proteins in as native an environment as possible to present the most relevant structures to the host humoral immune system. As shown here, however, if one uses an oil-in-water emulsifying adjuvant that also targets TLR-4, there is not a large benefit to the addition of the C3d elements in a fusion protein. A slight benefit in gp120-specific IgG secondary antibody elicitation was observed by gp120ΔC1/C5$(C3d)_2$ priming followed by two gp120ΔC1/C5 boosts compared to using either protein alone for all three inoculations. Since this experiment is internally well controlled for protein dose, we interpret these minor differences to be slight but meaningful. The gp120-C3d likely functions more efficiently as a prime even in the presence of Ribi, and then boosting without C3d may serve to focus the immune response toward gp120-specific elements and not divert potential antigenic interaction to non-primed or non-gp120-specific cells bearing CD21 or other C3d-binding proteins (Haas et al., 2004). Since this study utilized two fused repeats of C3d, it is possible that greater numbers of C3d repeats, or at lower protein doses, or if C3d is used on trimeric gp140 constructs as was recently done (Bower et al., 2004a, 2004b), there might be a greater benefit even in the presence of a classical oil-in-water adjuvant. We conclude that C3d may merit consideration for selected uses in candidate HIV-1 immunogens and that generally the activation of innate immunity through multiple pathways merits further investigation.

Materials and methods

Plasmid construction, transient expression and establishment of stable insect lines

Deletions of the N- and C-termini of gp120 were performed by PCR amplification and in-frame cloning into a C3d expression vector to create a gp120$(C3d)_2$ fusion protein as follows. To introduce the deletions, gp120 coding sequences were PCR amplified utilizing homologous forward and reverse primers designed to eliminate coding sequences for the first 82 residues from the N-terminus of gp120 and the last 19 amino acids from the C-terminus of gp120 (gp120ΔC1/C5). Purified PCR products were then directionally cloned into a gp120 mammalian expression vector, pSVIII (Helseth et al., 1990), in which expression is driven in a rev- and tat-dependent manner from a mini LTR. Tat is provided in trans by the co-transfection of the pSVtat expression plasmid. Previous analysis of this gp120 variant transiently expressed in 293T cells had indicated that this protein was recognized by a set of conformationally sensitive antibodies and bound CD4 with wild-type affinity (Wyatt et al., 1997).

To create a gp120ΔC1/C5$(C3d)_2$ fusion protein, the mammalian expressor plasmid pSG5$(C3d)_2$ was kindly provided by Drs. Douglas Fearon and Paul Dempsey (Dempsey et al., 1996) and contains two repeats of the murine C3d coding sequence in a continuous open reading frame. Immediately 5′ to the C3d coding sequences was a *Bgl*II restriction site that allowed in-frame cloning of gp120 coding sequences. Located between the two C3d coding cassettes were sequences encoding a flexible $(Gly_4Ser)_2$ linker designed to permit independent interaction of each C3d subunit with a CD21 molecule. Sequences coding for the gp120ΔC1/ΔC5 were PCR amplified from the pSVIII molecular clone utilizing primers possessing *Bgl*II sites and shuttled into the pSG5$(C3d)_2$ vector in the proper orientation. To make recombinant gp120ΔC1/C5$(C3d)_2$ fusion proteins, coding sequences were directionally subcloned from the pSG5$(C3d)_2$ shuttle vector into the pMtdel0 plasmid (Culp et al., 1991; Ivey-Hoyle et al., 1991). The pMt plasmid drives expression from the inducible *Drosophila* metallothionein promoter when transfected into *Drosophila* Schneider 2 (S2) cells and induced by heavy metals (Culp et al., 1991). Stable S2 producer cell lines were established by co-transfection with a hygromycin resistance plasmid and selection in hygromycin-containing media (Culp et al., 1991; Ivey-Hoyle et al., 1991).

Protein characterization and purification

To produce quantities of proteins sufficient for immunogenicity studies, and following selection of stably producing *Drosophila* S2 cell lines, expression of the gp120ΔC1/C5 and the gp120ΔC1/C5$(C3d)_2$ fusion proteins were induced by the addition of 750 um CuSO4 to the media. The proteins were purified to near homogeneity by affinity chromatography using an F105 affinity column as previously described (Wu et al., 1996). The F105 antibody recognizes a discontinuous gp120 epitope overlapping the CD4 binding site and thus selects for properly folded glycoproteins. The concentration of each protein was determined by optical density at 280 nm and by SDS gels to confirm purity and that the calculated concentrations were accurate (Fig. 2A). To confirm the gp120 structural integrity, glycoproteins were radiolabeled with ^{125}I by the lactoperoxidase method as described previously (Wu et al., 1996) and immunoprecipitations were performed by the addition of AIDS patient sera,

two gp120-specific antibodies (F105 and 17b) and with sCD4 in combination with a rabbit anti-CD4 antibody followed by the addition of protein A sepharose (Fig. 2B). Two polyclonal sera against murine C3 and human C3d were also included in the immunoprecipitation analysis (Fig. 2B).

To confirm that the C3d was functional, we incubated 125-labeled gp120ΔC1/C5 or gp120ΔC1/C5(C3d)$_2$ with CD21$^+$ Raji B cells in complete media for 1 h at RT. Following extensive washing, the cells were lysed in NP-40 buffer, immunoprecipitated with HIV patient serum and analyzed by SDS gels and autoradiography (Fig. 2C).

Inoculation of mice with selected variant gp120 glycoproteins

Groups of mice were immunized with 20 μg of selected gp120 variant glycoproteins by the subcutaneous route in the presence of either PBS (no adjuvant) or Ribi Adjuvant System (RAS; Sigma or Corixa). The Ribi formulation used here, and recommended by the manufacture for use in mice with a diverse range of immunogens (R-700, Corixa), contains squalene oil, polysorbate 80, synthetic trehalose dicorynomycolate (TDM) and MPL. Approximately 28 days apart, either three or four inoculations were administered and 7–10 days after each inoculation retro-orbital test bleeds were obtained. The serum isolated from the bleeds was analyzed for gp120 recognition by ELISA with either gp120ΔC1/C5 or gp120ΔC1/C5(C3d)$_2$ proteins coated on the plates.

ELISA gp120 binding assays

To detect antibodies capable of binding to HIV-1 gp120, the gp120ΔC1/C5 or gp120 ΔC1/C5(C3d)$_2$ proteins were coated overnight onto 96-well ELISA plates at 200 ng/well in 0.1 M sodium carbonate–bicarbonate buffer, pH 9.6. Following a wash to remove unbound proteins, the wells were incubated with blocking buffer (bb) that was composed of PBS containing 5% heat-inactivated fetal calf serum and 2% non-fat dried milk (Carnation) for 2 h at room temperature (RT). The sera were serially diluted in bb and incubated for 2 h at RT on the ELISA plate and following extensive washing in PBS with 2% TWEEN, anti-mouse horseradish peroxidase was added to all wells for 1 h at RT. Following a second extensive wash, 100 μl of substrate was added and the colorimetric reaction was stopped by adding an equal volume of 1 N hydrochloric acid. Optical density readings at 450 nM were used to detect binding antibodies to the gp120 variant proteins. For ease of comparison between ELISAs, the data were normalized and plotted as relative absorbance.

Acknowledgments

MK and JF contributed equally to this work. We would like to thank Liz Desjardins for excellent technical assistance and support. We are grateful to Dr. Marshal Posner for the F105 antibody and to Dr. James Robinson for providing the 17b antibody. We would like to thank Toni Garrison and Brenda Hartman for help with the figures. This work was supported in part by the NIH grant A142719-02.

References

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., Berger, E.A., 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272 (5270), 1955–1958.

Baldridge, J.R., McGowan, P., Evans, J.T., Cluff, C., Mossman, S., Johnson, D., Persing, D., 2004. Taking a toll on human disease: toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Expert Opin. Biol. Ther. 4 (7), 1129–1138.

Binley, J.M., Sanders, R.W., Clas, B., Schuelke, N., Master, A., Guo, Y., Kajumo, F., Anselma, D.J., Maddon, P.J., Olson, W.C., Moore, J.P., 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. J. Virol. 74 (2), 627–643.

Bower, J.F., Green, T.D., Ross, T.M., 2004a. DNA vaccines expressing soluble CD4-envelope proteins fused to C3d elicit cross-reactive neutralizing antibodies to HIV-1. Virology 328 (2), 292–300.

Bower, J.F., Yang, X., Sodroski, J., Ross, T.M., 2004b. Elicitation of neutralizing antibodies with DNA vaccines expressing soluble stabilized human immunodeficiency virus type 1 envelope glycoprotein trimers conjugated to C3d. J. Virol. 78 (9), 4710–4719.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., Sodroski, J., 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85 (7), 1135–1148.

Culp, J.S., Johansen, H., Hellmig, B., Beck, J., Matthews, T.J., Delers, A., Rosenberg, M., 1991. Regulated expression allows high level production and secretion of HIV-1 gp120 envelope glycoprotein in *Drosophila* Schneider cells. Biotechnology 9 (2), 173–177 (New York).

Dempsey, P.W., Allison, M.E., Akkaraju, S., Goodnow, C.C., Fearon, D.T., 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science 271 (5247), 348–350.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., Landau, N.R., 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381 (6584), 661–666.

Doranz, B.J., Rucker, J., Yi, Y., Smyth, R.J., Samson, M., Peiper, S.C., Parmentier, M., Collman, R.G., Doms, R.W., 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 85 (7), 1149–1158.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., Paxton, W.A., 1996. HIV-1 entry into CD4$^+$ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381 (6584), 667–673.

Feng, Y., Broder, C.C., Kennedy, P.E., Berger, E.A., 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272 (5263), 872–877.

Flynn, N.M., Forthal, D.N., Harro, C.D., Judson, F.N., Mayer, K.H., Para, M.F., 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J. Infect. Dis. 191 (5), 654–665.

Gao, F., Weaver, E.A., Lu, Z., Li, Y., Liao, H.X., Ma, B., Alam, S.M., Scearce, R.M., Sutherland, L.L., Yu, J.S., Decker, J.M., Shaw, G.M., Montefiori, D.C., Korber, B.T., Hahn, B.H., Haynes, B.F., 2005.

Antigenicity and immunogenicity of a synthetic human immunodeficiency virus type 1 group m consensus envelope glycoprotein. J. Virol. 79 (2), 1154–1163.

Green, T.D., Montefiori, D.C., Ross, T.M., 2003. Enhancement of antibodies to the human immunodeficiency virus type 1 envelope by using the molecular adjuvant C3d. J. Virol. 77 (3), 2046–2055.

Grundner, C., Pancera, M., Kang, J.M., Koch, M., Sodroski, J., Wyatt, R., 2004. Factors limiting the immunogenicity of HIV-1 gp120 envelope glycoproteins. Virology 330 (1), 233–248.

Haas, K.M., Toapanta, F.R., Oliver, J.A., Poe, J.C., Weis, J.H., Karp, D.R., Bower, J.F., Ross, T.M., Tedder, T.F., 2004. Cutting edge: C3d functions as a molecular adjuvant in the absence of CD21/35 expression. J. Immunol. 172 (10), 5833–5837.

Helseth, E., Kowalski, M., Gabuzda, D., Olshevsky, U., Haseltine, W., Sodroski, J., 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J. Virol. 64 (5), 2416–2420.

Ivey-Hoyle, M., Culp, J.S., Chaikin, M.A., Hellmig, B.D., Matthews, T.J., Sweet, R.W., Rosenberg, M., 1991. Envelope glycoproteins from biologically diverse isolates of immunodeficiency viruses have widely different affinities for CD4. Proc. Natl. Acad. Sci. U.S.A. 88 (2), 512–516.

Liu, F., Mboudjeka, I., Shen, S., Chou, T.H., Wang, S., Ross, T.M., Lu, S., 2004. Independent but not synergistic enhancement to the immunogenicity of DNA vaccine expressing HIV-1 gp120 glycoprotein by codon optimization and C3d fusion in a mouse model. Vaccine 22 (13–14), 1764–1772.

Miller, S.I., Ernst, R.K., Bader, M.W., 2005. LPS, TLR4 and infectious disease diversity. Nat. Rev., Microbiol. 3 (1), 36–46.

Molina, H., Wong, W., Kinoshita, T., Brenner, C., Foley, S., Holers, V.M., 1992. Distinct receptor and regulatory properties of recombinant mouse complement receptor 1 (CR1) and Crry, the two genetic homologues of human CR1. J. Exp. Med. 175 (1), 121–129.

Pancera, M., Wyatt, R., 2005. Selective recognition of oligomeric HIV-1 primary isolate envelope glycoproteins by potently neutralizing ligands requires efficient precursor cleavage. Virology 332 (1), 145–156.

Pantaleo, G., Koup, R.A., 2004. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. Nat. Med. 10 (8), 806–810.

Ross, T.M., Xu, Y., Green, T.D., Montefiori, D.C., Robinson, H.L., 2001. Enhanced avidity maturation of antibody to human immunodeficiency virus envelope: DNA vaccination with gp120-C3d fusion proteins. AIDS Res. Hum. Retrovir. 17 (9), 829–835.

Sanders, R.W., Vesanen, M., Schuelke, N., Master, A., Schiffner, L., Kalyanaraman, R., Paluch, M., Berkhout, B., Maddon, P.J., Olson, W.C., Lu, M., Moore, J.P., 2002. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. J. Virol. 76 (17), 8875–8889.

Srivastava, I.K., Stamatatos, L., Kan, E., Vajdy, M., Lian, Y., Hilt, S., Martin, L., Vita, C., Zhu, P., Roux, K.H., Vojtech, L., Montefiori, D., Donnelly, J., Ulmer, J.B., Barnett, S.W., 2003a. Purification, characterization, and immunogenicity of a soluble trimeric envelope protein containing a partial deletion of the V2 loop derived from SF162, an R5-tropic human immunodeficiency virus type 1 isolate. J. Virol. 77 (20), 11244–11259.

Srivastava, I.K., VanDorsten, K., Vojtech, L., Barnett, S.W., Stamatatos, L., 2003b. Changes in the immunogenic properties of soluble gp140 human immunodeficiency virus envelope constructs upon partial deletion of the second hypervariable region. J. Virol. 77 (4), 2310–2320.

Wu, L., Gerard, N.P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A.A., Desjardin, E., Newman, W., Gerard, C., Sodroski, J., 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. Nature 384 (6605), 179–183.

Wyatt, R., Sodroski, J., 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280 (5371), 1884–1888.

Wyatt, R., Desjardin, E., Olshevsky, U., Nixon, C., Binley, J., Olshevsky, V., Sodroski, J., 1997. Analysis of the interaction of the human immunodeficiency virus type 1 gp120 envelope glycoprotein with the gp41 transmembrane glycoprotein. J. Virol. 71 (12), 9722–9731.

Wyatt, R., Kwong, P.D., Desjardins, E., Sweet, R.W., Robinson, J., Hendrickson, W.A., Sodroski, J.G., 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature 393 (6686), 705–711.

Yang, X., Farzan, M., Wyatt, R., Sodroski, J., 2000a. Characterization of stable, soluble trimers containing complete ectodomains of human immunodeficiency virus type 1 envelope glycoproteins. J. Virol. 74 (12), 5716–5725.

Yang, X., Florin, L., Farzan, M., Kolchinsky, P., Kwong, P.D., Sodroski, J., Wyatt, R., 2000b. Modifications that stabilize human immunodeficiency virus envelope glycoprotein trimers in solution. J. Virol. 74 (10), 4746–4754.

Yang, X., Wyatt, R., Sodroski, J., 2001. Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. J. Virol. 75 (3), 1165–1171.

Yang, X., Lee, J., Mahony, E.M., Kwong, P.D., Wyatt, R., Sodroski, J., 2002. Highly stable trimers formed by human immunodeficiency virus type 1 envelope glycoproteins fused with the trimeric motif of T4 bacteriophage fibritin. J. Virol. 76 (9), 4634–4642.